Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Parainfluenza 3 Virus in Vaccines

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* assay method which employs a cell culture system utilizing cytopathic effect (CPE) and/or hemadsorption (HAd) of guinea pig red blood cells (RBC) to determine the parainfluenza 3 virus (PI3V) content of modified-live veterinary vaccines.

1.2 Keywords

Parainfluenza 3 virus; PI3V; potency test, titration, *in vitro*, CPE, HAd

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36^0 ± 2^{0}C, high humidity, 5% ± 1% CO_2

2.1.2 Water bath,[2] 37^0 ± 1^{0}C

2.1.3 Pipettors,[3] 25 µl, 500 µl, and tips[4]

2.1.4 Vortex mixer[5]

2.1.5 Multichannel pipettor, 50-300 µl x 8 or 12 channel[6]

2.1.6 Microscope,[7] inverted light

[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750-0649 or equivalent
[2] Model MW-1120A, Blue M Electric Co., 304 Hart St., Watertown, WI 53094 or equivalent
[3] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[4] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY, 11571 or equivalent
[5] Vortex-3 Genie, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716 or equivalent
[6] Finnipette, Labsystems OY, Pulttitie 9, 00810 Helsinki 81, Finland or equivalent
[7] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

2.1.7 Centrifuge[8] and rotor[9]

2.2 Reagent/supplies

2.2.1 PI3V Reference[10]

2.2.2 Madin-Darby bovine kidney-A cells[11] (MDBK-A) found to be free of extraneous agents as tested by Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Diluent Medium

2.2.3.1 9.61 g minimum essential medium with Earle's salts without bicarbonate[12]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[13]

2.2.3.3 Dissolve with 900 ml deionized water (DW).

2.2.3.4 Add 5.0 g lactalbumin hydrolysate or edamine[14] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to ingredients in **Section 2.2.3.3** with constant mixing.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[15]

2.2.3.6 Sterilize through a 0.22-µm filter.[16]

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[8] Model J6-B, Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92834-3100 or equivalent
[9] Model JS-4.0, Beckman Instruments, Inc. or equivalent
[10] Seed quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[11] Cat. No. ATCC CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776
[12] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[13] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[14] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[15] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[16] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine[17]
2. 100 units/ml penicillin[18]
3. 50 µg/ml gentamicin sulfate[19]
4. 100 µg/ml streptomycin[20]
5. 2.5 µg/ml amphotericin B[21]

2.2.3.8 Store at $4^{o} \pm 2^{o}C$

2.2.4 Growth Medium

2.2.4.1 900 ml of Diluent Medium

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.4.3 Store at $4^{0} \pm 2^{0}C$.

2.2.5 Maintenance Medium

2.2.5.1 980 ml Diluent Medium

2.2.5.2 Aseptically add 20 ml gamma-irradiated FBS.

2.2.5.3 Store at $4^{0} \pm 2^{0}C$.

2.2.6 Alsever's Solution

2.2.6.1 20.5 g dextrose ($C_6H_{12}O_6$)[22]

2.2.6.2 8.0 g sodium citrate ($Na_3C_6H_5O_72H_2$)[23]

2.2.6.3 4.2 g sodium chloride (NaCl)[24]

[17] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[18] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[19] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[20] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[21] Cat. No. A-4888, Sigma Chemical Co. or equivalent
[22] Cat. No. D12-500, Fisher Scientific Co., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent
[23] Cat. No. S279-500, Fisher Scientific Co. or equivalent
[24] Cat. No. 3624-01, J.T. Baker, Inc. or equivalent

2.2.6.4 0.55 g citric acid ($C_6H_8O_7$)[25]

2.2.6.5 Q.S. to 100 ml with DW.

2.2.6.6 Filter through a 0.22-µm filter.

2.2.6.7 Store at 4⁰ ± 2⁰C.

2.2.7 Phosphate buffered saline 10X (PBS)

2.2.7.1 8.0 g NaCl

2.2.7.2 0.2 g potassium chloride (KCl)[26]

2.2.7.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[27]

2.2.7.4 1.15 g sodium phosphate dibasic, anhydrous (Na_2HPO_4)[28]

2.2.7.5 Q.S. to 1000 ml with DW, adjust the pH to 7.0-7.3 with 5N sodium hydroxide (NaOH)[29]; autoclave at 15 psi, 121⁰ ± 2⁰C for 35 ± 5 min.

2.2.7.6 Store at 4⁰ ± 2⁰C

2.2.8 1X PBS

2.2.8.1 100 ml 10X PBS

2.2.8.2 900 ml DW

2.2.8.3 Store at 4⁰ ± 2⁰C.

2.2.9 Guinea pig (RBC) in an equal volume of Alsever's Solution

[25] Cat. No. A104-500, Fisher Scientific Co. or equivalent
[26] Cat. No. P217-500, Fisher Scientific Co. or equivalent
[27] Cat. No. 3246-01, J.T. Baker, Inc. or equivalent
[28] Cat. No. 3828-01, J.T. Baker, Inc. or equivalent
[29] Cat. No. SS256-500, Fisher Scientific Co. or equivalent

2.2.10 Trypsin versene (TV) Solution

2.2.10.1 8.0 g NaCl

2.2.10.2 0.40 g KCl

2.2.10.3 0.58 g $NaHCO_3$

2.2.10.4 0.50 g irradiated trypsin[30]

2.2.10.5 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)[31]

2.2.10.6 1.0 g dextrose

2.2.10.7 0.4 ml 0.5% phenol red[32]

2.2.10.8 Q.S. with DW to 1000 ml.

2.2.10.9 pH to 7.3 with $NaHCO_3$.

2.2.10.10 Filter through a 0.22-µm filter.

2.2.10.11 Store at $-20^0 \pm 4^0$C.

2.2.11 Tissue culture plates, 96 well[33]

2.2.12 Polystyrene tubes, 17 x 100 mm[34]

2.2.13 Polystyrene tubes, 12 x 75 mm[35]

2.2.14 Conical tube, 50 ml[36]

2.2.15 Serological pipette, 10 ml[37]

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[30] Cat. No. 0152-15-9, DIFCO Laboratories, P.O. Box 331058, Detroit, MI 48232-0758
[31] Cat. No. E 5134, Sigma Chemical Co. or equivalent
[32] Cat. No. P0290, Sigma Chemical Co. or equivalent
[33] Cat. No. 3596, Corning Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[34] Falcon® 2057, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
[35] Falcon® 2058, Becton Dickinson Labware or equivalent
[36] Cat. No. 62.547, Sarstedt, Inc., P.O. Box 468, Newton, NC 28658-0468 or equivalent
[37] Falcon® 7530, Becton Dickinson Labware, or equivalent

2.2.16 Graduated cylinders, 25 ml, 50 ml, 100 ml, and 250 ml[38], sterile

2.2.17 Infectious bovine rhinotracheitis monospecific antiserum[39] (IBR AS)

2.2.18 Bovine viral diarrhea monospecific antiserum[39] (BVD AS)

2.2.19 Bovine respiratory syncytial virus monospecific antiserum[39] (BRSV AS)

2.2.20 Plastic wash bottle, 500 ml[40]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in cell culture technique, the principles of aseptic technique, and virus titration assays.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set the water bath at $36^0 \pm 2^0$C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of MDBK-A Plates.

[38] Cat. No P34546-02, P34546-03, P34546-04, and P34546-05 respectively, Cole-Parmer Instrument Co., 625 Bunker Court, Vernon Hills, IL 60061-9872 or equivalent
[39] Reference quantities available upon request from the CVB-L or equivalent
[40] Cat. No. 2402, Nalge Nunc Int., 75 Panorama Creek Dr., Rochester, NY 14602 or equivalent

3.3.1.1 Cells are prepared from healthy, confluent MDBK-A cells, that are maintained by passing every 5 ± 2 days. Two days prior to test initiation, cells are removed from the growth containers by using TV Solution. Using a multichannel pipettor, add 200 µl/well of $10^{5.4}$ to $10^{5.6}$ cells/ml cells suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 MDBK-A plate for the controls and the first 3 Test Serials. Each additional plate allows testing of 4 Test Serials. These become the MDBK-A Plates. Incubate at 36° ± 2°C in a CO_2 incubator for 48 ± 12 hr. Growth Medium is not changed unless excess acidity occurs or cells are not confluent in 48 hr.

3.3.2 Preparation of PI3V Reference Control.

3.3.2.1 On the day of test initiation, a vial of PI3V Reference is rapidly thawed in a $36^0 \pm 2^0$C water bath and tenfold dilutions made:

1. Place 4.5 ml of Diluent Medium into 6, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-6} respectively, using a 10-ml serological pipette.

2. Using a 500 µl pipettor, transfer 500 µl of PI3V Reference to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

3. Using a new pipette tip, transfer 500 µl from the 10^{-1} labeled tube to the 10^{-2} tube; mix by vortexing.

4. Repeat **Section 3.3.2.1.3** for each of the subsequent dilutions, transferring 500 µl from the previous dilution to the next dilution tube until the dilution series is completed.

3.3.3 Preparation of 0.5% RBC suspension for HAd test

1. Upon receipt of the guinea pig blood, transfer 20 ml of RBCs to a 50-ml conical tube.

2. Q.S. to 50 ml with Alsevers Solution.

3. Mix by inverting several times.

4. Centrifuge for 15 ± 5 min at 400 x g (1500 rpm in the J6B centrifuge with a JS-4.0 rotor).

5. Remove supernatant and buffy coat by aspirating with a 10-ml serological pipette.

6. Repeat **Sections 3.3.3.2 through 3.3.3.4** for a total of 3 washes.

7. Pipette 500 µl of packed RBCs to 100 ml of 1X PBS; mix by inverting for a 0.5% RBC suspension.

8. Store at 4^0 ± 2^0C; use within 1 wk of collection of RBCs.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of test initiation, remove the seal and stopper from both the Test Serial bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer's instructions (e.g. for 50 dose container of 2 ml per dose, reconstitute with 100 ml of diluent) and aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

3.4.2 Viral neutralization. In order to determine the PI3V titer in a multifraction product, neutralize the IBR, BVD, and BRSV fractions with monospecific antiserum.

3.4.2.1 IBR/PI3V Vaccine

1. 1.0 ml of the reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. Mix 500 µl of **Section 3.4.2.1.1** dilution of Test Serial with 500 µl IBR AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at room temperature (RT) ($23^{0} \pm 2^{0}$C) for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.2 IBR/PI3V/BVD Vaccine

1. 1.0 ml of reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. 1.0 ml of **Section 3.4.2.2.1** dilution of Test Serial is mixed with 500 µl of IBR AS and 500 µl of BVD AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.3 IBR/PI3V/BVD/BRSV Vaccine

1. 2.0 ml of reconstituted Test Serial is diluted with 8.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. 1.5 ml of **Section 3.4.2.3.1** dilution of the Test Serial is mixed with 500 µl of IBR AS, 500 µl of BVD AS and 500 µl of BRSV AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.4 PI3V Monovalent Vaccine

1. 500 µl of the reconstituted Test Serial is diluted with 4.5 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3.4.3 Sample dilutions. Five, tenfold dilutions are made from the 10^{-1} dilution of the Test Serial using Diluent Medium.

1. Place 4.5 ml of Diluent Medium into each of five 17 x 100-mm polystyrene tubes labeled 10^{-2} through 10^{-6}, using a 10-ml serological pipette.

2. Pipet 500 µl of the Test Serial from the 10^{-1} tube into the 10^{-2} tube; mix by vortexing. Discard pipette tip.

3. Using a new tip each time repeat **Section 3.4.3.2** to the remaining tubes transferring 500 µl from the previous dilution tube to the next tube until the final dilution is made (10^{-6}); mix by vortexing between each dilution.

4. Performance of the test

4.1 Aseptically decant the Growth Medium from the MDBK-A Plates into a suitable container.

4.2 Inoculate 5 wells/dilution with 25 µl/well of the diluted Test Serial and the PI3V Reference Control (10^{-3} through 10^{-6}). Change tips between each unique sample (e.g., each Test Serial and the PI3V Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}). This becomes the MDBK-A Test Plate.

4.3 Maintain 5 wells as uninoculated cell culture controls on each plate.

4.4 Incubate the MDBK-A Test Plate at $36^0 \pm 2^0$C in a CO_2 incubator for 60 ± 10 min for virus adsorption.

4.5 After incubation, add 200 µl/well of Maintenance Medium to the MDBK-A Test Plates with a multichannel pipettor.

4.6 Incubate the MDBK-A Test Plate undisturbed at $36^0 \pm 2^0$C in a CO_2 incubator for 102 ± 6 hr.

4.7 At the end of incubation, read the MDBK-A Test Plate at 100X magnification on an inverted light microscope and examine for CPE characterized by cell fusion.

4.7.1 Wells displaying 1 or more CPE foci, are considered to be positive for PI3V.

4.7.2 Results are recorded as the number of CPE positive wells versus the total number of wells examined for each dilution of the Test Serial and the PI3V Reference Control.

4.8 If CPE is not detected by microscopic examination, the MDBK-A Test Plate may be read by HAd as follows:

4.8.1 Maintenance Medium is decanted from the MDBK-A Test Plate into a suitable autoclavable container and cells are rinsed once with RT 1X PBS using a plastic wash bottle or by immersion into a pan filled with RT 1X PBS. 1X PBS is decanted immediately after filling.

4.8.2 To each well add 200 µl of 0.5% RBC Suspension.

4.8.3 Plates are allowed to incubate for 15 ± 5 min at RT.

4.8.4 The 0.5% RBC Suspension is decanted and the cell monolayer washed 3 times with 1X PBS as in **Section 4.8.1**.

4.8.5 The final wash is decanted and the monolayers examined using an inverted light microscope at 100X magnification. Wells containing one or more RBC clusters adhering to the cell monolayer are considered to be positive for PI3V.

4.8.6 Record the number of HAd positive wells versus the total number of wells examined for each dilution of the Test Serial and the PI3V Reference Control.

4.9 Calculate the PI3V endpoints of the Test Serial and the PI3V Reference Control using the method of Spearman-Kärber as commonly modified. The titers are expressed as $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) of the test wells.

Example:

10^{-3} dilution of Test Serial = 5/5 wells CPE/HAd positive
10^{-4} dilution of Test Serial = 5/5 wells CPE/HAd positive
10^{-5} dilution of Test Serial = 2/5 wells CPE/HAd positive
10^{-6} dilution of Test Serial = 0/5 wells CPE/HAd positive

Test dose titer = (X - d/2 + [d * S]) where:

X = $\log_{10}$ of lowest dilution (3)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportion of CPE/HAd positive

$$\frac{(5 + 5 + 2)}{5} = \frac{12}{5} = 2.4$$

Test dose titer = (3 - 1/2 + (1 * 2.4) = 4.9

Adjust the titer to the Test Serial dose size by adding the $\log_{10}$ of the reciprocal of the Inoculation Dose divided by the Test Serial Dose where:

Inoculation Dose = amount of diluted Test Serial added to each well of the Test Plate

Test Serial Dose = Manufacutrer's recommended vaccination dose

Example:

PI3 endpoint = 4.9

$\frac{0.025 \text{ ml inoculum}}{2 \text{ ml dose}} = \frac{1}{80}$ = 1.9 log

Total = 6.8 log

Titer of the Test Serial is $10^{6.8}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 For a valid assay

5.1.1 The calculated $TCID_{50}$ titer of the PI3V Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell controls can not exhibit any CPE or cloudy media that would indicate contamination.

5.1.3 The lowest dilution of the PI3V Reference Control must exhibit a 100% positive CPE/HAd (5/5), and the highest (most dilute) must exhibit no positive CPE/HAd (0/5).

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production (OP), the Test Serial is considered **SATISFACTORY**.

5.4 If the validity requirements are met but the titer of the Test Serial is less than the titer contained in the APHIS filed Outline of Production, the Test Serial is retested according to 9 CFR, Part 113.8.

6. Report of test results

6.1 Results are reported as $TCID_{50}$ per dose.

6.2 Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.309, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral GE, (Ed.), 1978, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg.731.

7.2 Finney, DJ, 1978, *Statistical method in biological assay*. Griffin, London. 3rd edition, pg.508.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, to provide additional detail and to reflect these changes from the superseded protocol:

1) the use of Madin-Darby bovine kidney-A cells instead of primary bovine embryonic kidney cell cultures

2) the replacement of the 24-well cell culture plates by 96-well plates, and

3) to include newly added vaccine viruses to the serum neutralization step.